Beneficial effects of alkaline phosphatase in septic shock

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**Objective:** Alkaline phosphatase may decrease the harmful effects of lipopolysaccharide by detoxifying lipid A. The aim of this study was to investigate whether administration of alkaline phosphatase is beneficial in a clinically relevant septic shock model.

**Design:** Interventional laboratory study.

**Setting:** University hospital animal research laboratory.

**Subjects:** Fourteen fasted, anesthetized, invasively monitored, mechanically ventilated, female sheep (27.6 ± 3.9 kg).

**Interventions:** Each animal received 1.5 g/kg body weight of feces intraperitoneally to induce sepsis. Ringer’s lactate and a 6% hydroxyethyl starch solution were infused throughout the experiment to prevent hypovolemia. Two hours after feces injection, animals were randomized to alkaline phosphatase (60 units/kg intravenous bolus followed by a continuous infusion of 20 units/kg/hr for a total of 15 hrs) or no alkaline phosphatase (control group).

**Measurements and Main Results:** All animals were studied until their spontaneous death or for a maximum of 30 hrs. Plasma alkaline phosphatase concentrations decreased in the control group but increased in the treatment group following alkaline phosphatase administration. In the treatment group, the PaO2/FiO2 ratio was higher (p < .05), blood interleukin-6 concentrations were lower (p < .05), and the survival time was longer (median time 23.8 vs. 17.0 hrs, p < 0.05) than in the control group. There were no significant differences in systemic hemodynamics or diuresis.

**Conclusions:** In this clinically relevant septic shock model, alkaline phosphatase administration improved gas exchange, decreased interleukin-6 concentrations, and prolonged survival time. (Crit Care Med 2006; 34:2182–2187)

**Key Words:** peritonitis; lipopolysaccharide; gas exchange; sheep

Lipopolysaccharide (LPS) can be detected in the blood of up to 78% of patients with severe sepsis (1). This is associated with higher mortality rates, and removal of LPS has been viewed as a possible approach to improve patient outcome from sepsis (2). Alkaline phosphatase is a constitutive ecto-enzyme present in different tissues (3) with different activities. Alkaline phosphatase has several natural substrates, including phosphoethanolamine, inorganic phosphate, and pyridoxal 5-phosphate (4). Alkaline phosphatase can also convert lipid A (5), the main toxic part of LPS, to monophosphoryl lipid A, which is virtually nontoxic in vitro and in vivo (6). Pretreatment with monophosphoryl lipid A even reduced endotoxin-induced mortality in the rat (7).

Some studies in rodents have suggested a protective role of alkaline phosphatase in sepsis. Poelstra et al. (6, 8) demonstrated that renal alkaline phosphatase extracts limited the localized intradermal inflammatory reaction elicited by LPS injection in rats. These authors also reported that levamisole, an inhibitor of intestinal alkaline phosphatase, increased mortality in a rat intraperitoneal Escherichia coli sepsis model (6, 8). Other studies have indicated that exogenous administration of placental-alkaline phosphatase improves survival in mouse endotoxemia (9) and intraperitoneal E. coli models (10). However, recently van Veen et al. (11) showed that alkaline phosphatase administration did not improve survival in a mice model of cecal ligation and puncture (CLP), despite an attenuated inflammatory response.

The goal of the present study was to investigate the effect of administration of alkaline phosphatase on mortality and outcome in a clinically relevant model of septic shock secondary to peritonitis (12, 13).

**METHODS**

The study was conducted in accordance with the guidelines established by the Institutional Review Board for animal care of the Free University of Brussels. Care and handling of the animals were in accordance with National Institutes of Health guidelines (Institute of Laboratory Animal Resources). Fourteen female sheep (27.6 ± 3.9 kg) were fasted for 24 hrs with free access to water before the experiment.

**Instrumentation.** On the day of the experiment, the animals were initially weighed, premedicated with intramuscular midazolam (Dormicum, Roche, Attikis, Greece; 0.25 mg/kg) and ketamine hydrochloride (Imalgine, Merial, Lyon, France; 20 mg/kg), and placed in the supine position. The cephalic vein was cannulated with a peripheral venous 18-gauge catheter (Surflo IV Catheter, Terumo, Belgium). Following intravenous administration of fentanyl (Janssen, Berchem, Belgium; 30 μg/kg) and pancuronium bromide (Pavulon, Organon, Oss, the Netherlands; 0.1 mg/kg), the trachea was intubated (tracheal tube, 8.0; Hi-Contour, Mallinckrodt Medical, Ireland). Mechanical ventilation was started in controlled volume mode (Servo Ventilator 900 C, Siemens-Elema, Sweden) with a tidal volume of 9 mL/kg, a positive end-expiratory pressure of 5 cm H2O, an FiO2 of 1, an inspiratory-expiratory time of 1:2, and a square wave pattern. Respiratory rate was adjusted to maintain end-tidal carbon dioxide pressure (47210 A Capnometer, Boehringer, Germany) between 35 and 45 mm Hg. A 60-cm plastic tube (inner diameter 1.8 cm) was inserted into the stomach to drain its content and prevent reflux distension. A Foley catheter (14-Fr, Beiersdorf AG, Germany) was placed to measure urine output. The right femoral artery

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and vein were exposed under strict sterile conditions. An arterial catheter (6-Fr Vygon, Cirencester, UK) was introduced and connected to a pressure transducer (Edwards Life Sciences, Irvine, CA) zeroed at mid-thorax level. Through the femoral vein, an introducer was inserted and a 7-Fr. pulmonary artery flotation catheter (Edwards Life Sciences, Baxter, Irvine, CA) was advanced into the pulmonary artery under monitoring of pressure waveforms. A midline laparotomy was performed and, after cecotomy, 1.5 g/kg body weight of feces was collected. The cecum was closed and the area around the cut was disinfected with iodine. An additional pouch suture was performed to prevent contamination, and the cecum was returned to the abdominal cavity. A large plastic tube was inserted through the abdominal wall for later injection of feces. The abdomen was then closed in two layers. After the surgical preparation, animals were turned to the prone position and allowed to stabilize for 2 hrs. At the end of this period, baseline measurements were performed.

All sheep were sedated with intravenous ketamine 10 mg/kg/hr, morphine 0.5 mg/ (kg/hr), and midazolam 0.5 mg/kg/hr. Muscular blockade was achieved with pancuronium bromide 0.1 mg/kg/hr. Boluses of intravenous fentanyl 3 μg/kg/hr were administered if needed in order to prevent an increase in heart rate and arterial pressure due to insufficient anesthesia. After surgical operation, controlled mechanical ventilation was adjusted to ensure normoxia (80 mm Hg ≤ Paco2 ≤ 120 mm Hg) and normocapnia (35 mm Hg ≤ Paco2 ≤ 45 mm Hg) according to repeated blood gas analysis (ABL500 OSMS Radiometer, Copenhagen, Denmark). Hemoglobin concentration and oxygen saturation were measured with an analyzer calibrated for animals (OSMS Radiometer, Copenhagen, Denmark).

All monitored variables were recorded every 60 mins. Heart rate, mean arterial pressure, central venous pressure, pulmonary arterial pressure, and pulmonary arterial occlusion pressure measurements were referenced to mid-chest level and obtained at expiration (Sirecust 404 Siemens, Germany). Core temperature, cardiac output (Vigilance Baxter, Edwards Critical-Care), minute volume, plateau pressure, inspiratory tidal volume, and end-tidal carbon dioxide pressure were continuously monitored. Body surface area was calculated from the equation BSA = 0.084 × (body weight kg)0.23 (14). Cardiac index (L·min⁻¹·m²), stroke volume index (mL·mmHg⁻¹·m²), systemic vascular resistance index (dyne·sec·cm⁻⁵), pulmonary vascular resistance index (dyne·sec·cm⁻⁵), oxygen delivery (mL/kg·min), and oxygen consumption (mL/kg·min) were calculated using standard formulas.

After baseline measurements had been performed, feces were injected into the abdominal cavity followed by 120 mL of air to empty the tube. Two hours after feces injection, animals were then randomized to a treatment group that received intestinal alkaline phosphatase (kindly provided by PharmAAware, Utrecht, Netherlands) as an intravenous bolus of 60 units/kg followed by a continuous infusion of 20 units/kg/hr for 15 hrs (we selected this formulation based on the previous pilot study which showed that it would increase alkaline phosphatase levels to three times baseline levels) or a control group, which received no alkaline phosphatase. The alkaline phosphatase was given at 100 units/mL, which was <0.1% of the total fluid infused and represents only a few milligrams of total protein.

Core temperature was kept at a minimum of 36.5°C using heating pads. Hyperthermia was not treated (15). A 6% hydroxyethyl starch solution (MW 130,000, degree of substitution 0.6, Voluven, Fresenius, Bad Homburg, Germany) and a Ringer's lactate solution (volume ratio = 1:1) were initially infused at a rate of 2 mL/(kg/hr) each. Fluid resuscitation was titrated to maintain mean arterial pressure above 60 mm Hg, cardiac filling pressures and cardiac output at least at baseline levels, and hemocoagulation (defined as a hemoglobin level increased by ≥2 g/dL in 1 hr) throughout the experiment. Supplemental infusions (volume challenge) of Ringer's lactate 4 mL/kg + hydroxyethyl starch 4 mL/kg over 5 mins were given if the stroke volume decreased by >10% from baseline, mean arterial pressure fell below 60 mm Hg (defined as hypotension), or urine flow dropped below 0.5 mL/kg/hr (defined as oliguria). No antibiotics and no vasoactive agents were administered. Animals were observed until spontaneous death or up to 30 hrs after baseline.

Arterial samples were taken at baseline and 1, 2, 3, 7, 11, 15, and 19 hrs after feces injection. Arterial blood was sampled in heparinized syringes and centrifuged 3,000 rounds/min for 15 mins at 4°C. Plasma was extracted and saved at −78°C for later cytokine and alkaline phosphatase measurements. Colloid osmotic pressure was measured (Onkometer BMT 923, Berlin, Germany).

Mouse anti-ovine interleukin (IL)-6 monoclonal antibody (Serotec MCA1659, Kidlington, UK), rabbit anti-ovine IL-6 polyclonal antibody (Serotec AHP424), and sheep anti-rabbit horseradish peroxidase (HRP) conjugated antibody (Serotec STAR54, Kidlington, UK) were used to measure IL-6 levels. In short, the monoclonal antibody was used as a coating antibody with a concentration of 1:200, diluted in phosphate-buffered saline (PBS) and incubated overnight on 96-well enzyme-linked immunosorbent assay plates (Greiner) at 4°C. After discarding the coating solution, 250 μL of blocking buffer (PBS/1% BSA) was added for 2 hrs at room temperature and then rinsed three times with PBS/0.05% Tween. A 50-μL serum sample was diluted with 50 μL of PBS/1% BSA, placed in plate wells, and incubated for 1 hr at room temperature. The plates were then washed three times before adding detection polyclonal antibody (HRP-Ab) and incubated 1 hr at room temperature. After being rinsed three times, the substrate for the conjugated HRP was added to the plate and allowed to react for 10 mins. The optical density of the plate-wells was then read on an enzyme-linked immunosorbent assay plate reader at 450 nm.

For alkaline phosphatase measurement, the amount of alkaline phosphatase causing the hydrolysis of 1 μMol of paranitrophenyl phosphate per minute at pH 9.6 and 25°C was defined as 1 unit (16). The change in optical density at 405 nm per unit time is a measure of the alkaline phosphatase activity.

**Statistical Analysis.** Baseline characteristics of the animals are presented as mean ± so, and differences between groups were compared by Student’s t-test. The effect of the treatment was analyzed using a mixed-effects models analysis with treatment group, time, and subjects nested in group as factors. If significant, each time point difference between group animals was compared with a t-test with Bonferroni correction. Survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. Statistical tests were two-tailed, and a p < .05 was considered statistically significant. All statistical analyses were performed using JMP 0.0 statistical software (SAS Institute, Cary, NC).

**RESULTS**

There was no significant difference between the two groups in body weight or any other variable at baseline (Table 1). The fluid infusion rate, cardiac filling pressures, and colloid osmotic pressure were similar between groups during the entire observation period. Both groups received similar amounts of fluid. Full fluid resuscitation provoked a typical hyperdynamic septic shock suggested by the decreased mean arterial pressure, systemic vascular resistance, and increased cardiac index and blood lactate concentration (Fig. 1). There was no significant difference in any systemic hemodynamic variable or in diuresis.

The alkaline phosphatase concentration decreased sharply in both groups after injection of feces. Alkaline phosphatase concentrations increased more than three-fold in the treatment group following alkaline phosphatase administration, reaching statistical significance at 3 hrs (Fig. 2; p < .05). Interestingly, alkaline phosphatase levels decreased toward baseline values soon after cessation of the study drug. Plasma IL-6 levels increased in both groups and became significantly lower after 5 hrs in the treated animals (Fig. 3; p < .05).

The PaO2/FiO2 ratio was markedly higher in the alkaline phosphatase group compared with the control group and reached statistical significance from hour.

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6 \( (p < .05, \text{Fig. 4a}) \). In addition, there was a tendency for an improved compliance in the alkaline phosphatase group, but the difference did not reach statistical significance \( (p = .10, \text{Fig. 4b}) \).

The survival time was significantly prolonged in the animals treated with alkaline phosphatase than in the controls (median 23.8 vs. 17.0 hrs, \( p < .05, \text{Fig. 5} \)).

**DISCUSSION**

The main findings of this study are that the administration of alkaline phosphatase in septic shock due to fecal peritonitis improved gas exchange and prolonged survival time. These results support a potentially beneficial effect of alkaline phosphatase administration in delaying respiratory failure associated with severe sepsis and septic shock.

Our model attempts to reproduce the clinical condition as closely as possible. It reproduces a laparotomy under anesthesia and fecal peritonitis with prolonged administration of sedation and analgesia. Fluid resuscitation was optimized according to multiple clinical end points, including avoiding hypovolemia as suggested by hemoconcentration, hypotension, and oliguria. Fever and tachycardia were associated with a hyperdynamic pattern with arterial hypotension, elevated cardiac index, and decreased systemic vascular resistance and lactic acidosis, all clinical features of human septic shock. Our model has been shown to reproduce a polybacterial peritonitis \( (12) \), which is closely related to clinical peritonitis in humans. In the absence of antibiotic or vasopressor therapy, the mortality of our model is 86% after 24 hrs. The

<table>
<thead>
<tr>
<th>Variable</th>
<th>Alkaline Phosphatase Group</th>
<th>Control Group</th>
<th>( p ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>26.4 ± 1.7</td>
<td>28.8 ± 1.2</td>
<td>.27</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>38.7 ± 0.2</td>
<td>39.2 ± 0.4</td>
<td>.26</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>107 ± 4</td>
<td>107 ± 7</td>
<td>.53</td>
</tr>
<tr>
<td>Mean pulmonary arterial pressure, mm Hg</td>
<td>17 ± 2</td>
<td>15 ± 1</td>
<td>.2</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>131 ± 4</td>
<td>125 ± 7</td>
<td>.46</td>
</tr>
<tr>
<td>Cardiac index, L/min/m²</td>
<td>4.6 ± 0.4</td>
<td>4.9 ± 0.3</td>
<td>.52</td>
</tr>
<tr>
<td>( \text{PaO}_2/\text{FiO}_2 )</td>
<td>406 ± 36</td>
<td>398 ± 24</td>
<td>.85</td>
</tr>
<tr>
<td>Lactate, mmol/L</td>
<td>0.8 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>.22</td>
</tr>
</tbody>
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**Table 1. Baseline variables (mean ± sd)**

![Figure 1](https://example.com/figure1.png)

Figure 1. Evolution of mean arterial pressure (MAP; top left), cardiac index (CI; top right), systemic vascular resistance index (SVRI; bottom left), and blood lactate concentrations (bottom right) in the two groups (mean ± sd). Squares, alkaline phosphatase treatment group; circles, control group.
evolution to death is related to multiple organ failure characterized by hypotension, oliguria, and respiratory alkalosis.

Figure 2. Arterial blood alkaline phosphatase (AP) concentrations in the two groups. Squares, alkaline phosphatase treatment group; circles, control group. *p < .05.

Figure 3. Arterial blood interleukin (IL)-6 concentrations in the two groups. Squares, alkaline phosphatase treatment group; circles, control group. *p < .05.

A strength of our study design is the postinsult initiation of the treatment. Indeed, alkaline phosphatase significantly prolonged survival time despite a 2-hr delay in administration after feces injection when signs of systemic inflammatory response were already present. Our findings complement two previous studies of alkaline phosphatase infusion in rodents. Bentala et al. (9) showed a survival benefit (100% compared with 57% at day 6) in mice receiving 0.1 unit of placental alkaline phosphatase, but the treatment was introduced immediately following LPS administration, a strategy that would be difficult to achieve in a clinical setting. Similarly, Verweij et al. (10), using an intraperitoneal inoculation of bacteria, demonstrated a 24-hr survival rate of 100% compared with 50% for untreated animals, but again they administered alkaline phosphatase early, only 20 mins after the onset of peritonitis. In contrast to these results, van Veen et al. (11) found that pretreatment (5 mins before CLP) and early treatment (15 mins after CLP) with alkaline phosphatase did not improve survival in a mouse model of CLP. This may due to the fact that only a bolus intervention (0.15 units/g) was administered in that study. Indeed, after 4 hrs, serum alkaline phosphatase activity had returned to the baseline level, whereas the outcome was evaluated after 24 hrs. Another possible reason is that the model was not severe enough since a 23-gauge needle was used for the puncture.

Why exogenous alkaline phosphatase administration shows beneficial effects in septic shock is not entirely clear. Indeed, even the physiologic role of alkaline phosphatase had remained unexplained, largely due to its unphysiologic pH optimum above 10 (17), until Poelstra et al. (8) described its strong ability to detoxify LPS. This characteristic raised the possibility that alkaline phosphatase belongs to our natural defensive mechanism (6). Severe sepsis and septic shock are associated with capillary leakage that may contribute to interstitial edema (18). The resulting intestinal ischemia and impaired epithelial barrier function may predispose to translocation of bacterial products and further aggravate the situation (19, 20). Exogenous administration of alkaline phosphatase might prevent this further insult. Likewise, Koyama et al. (5) showed that, following oral LPS administration in rats, the addition of a phenylalanine, an inhibitor of intestinal alkaline phosphatase isoenzymes, results in a doubling of serum LPS concentration. Furthermore, the product of alkaline phosphatase with LPS, monophosphoryl lipid A, has been demonstrated to be an immune response modulator (21–23), and preconditioning of monophosphoryl lipid A prolonged the survival time in a pig endotoxin shock model (24).

An important finding of our study is that sepsis decreased endogenous alkaline phosphatase concentrations: The plasma alkaline phosphatase concentration decreased to 50% of baseline values as early as 3 hrs after feces injection. This observation is in line with that of Verweij et al. (10) showing that intravenous LPS administration decreased serum alkaline phosphatase concentrations in mice. This could be due to an increased consumption and/or a decreased production of alkaline phosphatase in sepsis. Knowing that LPS exposure can increase alkaline phosphatase expression in different tissues (25), the decrease in circulating alkaline phosphatase levels may be caused by increased alkaline phosphatase turn-
over. In this context, it has been proposed that LPS, or its combination with other molecules (like LPS-binding protein), when conjugated with alkaline phosphatase is transferred to macrophages (Kupffer cells) and cleared together with LPS (26). To further strengthen this hypothesis, in our study, when alkaline phosphatase administration was stopped after 15 hrs, serum alkaline phosphatase levels decreased sharply. This suggests that alkaline phosphatase production was insufficient to cope with the increased consumption.

We have no direct proof that the beneficial effects of alkaline phosphatase are due to its LPS detoxification effects in our model. The lower IL-6 levels in the alkaline phosphatase-treated animals suggested a decreased inflammatory response in this group. As a biomarker of inflammation and tissue injury (27), IL-6 is correlated with morbidity and mortality in critically ill patients (28–30). Similarly, van Veen et al. (11) demonstrated that bolus alkaline phosphatase administration resulted in lower blood IL-6 and tumor necrosis factor-α levels in a murine CLP model of polymicrobial sepsis.

We also observed a significant protective effect of alkaline phosphatase on the pulmonary function of these septic animals, as indicated by a significantly higher PaO2/FiO2 ratio and a somewhat better compliance in the alkaline phosphatase treatment group. Similarly, van Veen et al. (11) showed that alkaline phosphatase administration reduced lung damage in a mouse CLP model, as suggested by decreased neutrophil infiltration reflected by decreased myeloperoxidase levels. Abdominal sepsis is a common cause of acute respiratory distress syndrome, which is associated with a high mortality rate (31). The relation between an LPS insult and sepsis-induced respiratory failure has been demonstrated in animals (32) and humans (33). Clearly our model represents an extrapulmonary form of acute respiratory distress syndrome secondary to peritonitis, with increased capillary permeability and increased neutrophil infiltration (13). A possible explanation for the beneficial effect is that alkaline phosphatase may protect type I and II pneumocytes (25) and epithelial cell function (34) since LPS exposure increases alkaline phosphatase secretion from these cells. Another possible mechanism could be through a decrease in myeloperoxidase activity (11), as alkaline phosphatase administration has been shown to decrease neutrophil infiltration in bronchial alveolar lavage fluid following LPS stimulation in a mouse model (6, 9).

Our study has several limitations. First, the animals were young and initially healthy and their response may be different than older acutely ill patients with compromised cardiorespiratory reserve. Also, we provided optimal fluid management and full ventilatory support but chose not to
give antibiotics or vasoactive agents to avoid the influence of these additional variables and to obtain a lethal model. We cannot exclude that antibiotic administration could increase the release of LPS into the circulation and make the model more severe. We did not succeed in measuring blood LPS levels; however, these measurements are notoriously unreliable, and peritonitis is known to be associated with endotoxia (35). Another limitation is our use of only one dosage of alkaline phosphatase. We selected this formulation based on the finding that it increased alkaline phosphatase levels to three times baseline levels, and previous studies showed that these levels were protective in a mouse endotoxia model (6, 9, 10), but we cannot say from our results that a higher dosage would not be better or a lower dosage as good. In addition we perfused the drug for only 15 hrs. Since the serum level of alkaline phosphatase rapidly decreases after discontinuation of the drug we might have been able to show a greater difference if we had administered alkaline phosphatase for a longer period of time. Further studies using different doses and longer periods of administration could be of interest to better define the pharmacokinetics and effects of this drug during sepsis.

CONCLUSIONS

We conclude that in this clinically relevant septic shock model, alkaline phosphatase administration significantly improved gas exchange, decreased blood IL-6 levels, and prolonged survival time. Our results raise the possibility that alkaline phosphatase may be a useful intervention for the treatment of sepsis, especially for acute lung injury associated with sepsis. Based on the available experimental observations, a clinical phase II trial is ongoing in patients with sepsis and septic shock.

REFERENCES